Clonal Analysis of CD4$^+$/CD8$^+$ T Cells in a Patient with Aplastic Anemia

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Abstract

T cell clones were established from peripheral blood of a patient with severe aplastic anemia. 8 of 18 individual clonal T cell populations stably coexpressed CD4 and CD8 molecules, a phenotype characteristic for thymocytes and a minor subpopulation of circulating T lymphocytes. Analysis of T cell receptor genes revealed identical rearrangements of T cell receptor β chain genes, suggesting clonality of these T cells. CD4$^+$/CD8$^+$ T cells clones were found to be efficiently cytotoxic towards autologous lymphoblasts. Autocytotoxicity could be blocked by a CD3 MAb, a MAb specific for monomorphic MHC class II determinants, and particularly, by an MHC-DP-specific MAb, suggesting specificity for autologous DP molecules. Perhaps more important, CD4$^+$/CD8$^+$ T cell clones inhibited differentiation of autologous progenitor enriched bone marrow cells in vitro by a direct cell-mediated mechanism. These data suggest that circulating cytotoxic CD4$^+$/CD8$^+$ T cell clones specific for autologous MHC-DP determinants may be involved in hematopoietic failure in some cases of aplastic anemia. (*J. Clin. Invest.* 1991. 87:1567–1574.) Key words: T cell receptor • CD4$^+$/CD8$^+$ T cells • aplastic anemia

Introduction

Normal T lymphocyte differentiation results in two major subpopulations, CD4$^+$/CD8$^-$ helper/inducer and CD4$^-$/CD8$^+$ cytotoxic/suppressor T lymphocytes (1). These T cells express an α/β T cell receptor (TCR)[1] and recognize antigen in association with MHC-class I molecules (CD4$^-$/CD8$^+$ T cells) and class II molecules (CD4$^+$/CD8$^-$ T cells), respectively (2, 3). During T lymphocyte differentiation, CD4 and CD8 molecules are coexpressed on immature cortical thymocytes. It has been proposed that these CD4$^+$/CD8$^+$ thymocytes either give rise to mature CD4$^+$/CD8$^-$ and CD4$^-$/CD8$^+$ thymocytes or are eliminated during differentiation in the course of a process that leads to acquisition of self tolerance (4–7). Besides mature CD4$^+$/CD8$^-$ and CD4$^-$/CD8$^+$ T cells, a minor T cell subpopulation of 1–5% of peripheral blood T lymphocytes has been identified expressing neither CD4 nor CD8 molecules (8–10). These CD4$^-$/CD8$^-$ T cells of not well defined function express primarily a TCR-γ/δ in association with CD3. On the other hand, mature T lymphocytes coexpressing CD4 and CD8 molecules were found in low frequencies (0.5–8% of circulating T lymphocytes) in peripheral blood (11).

Acquired severe aplastic anemia consists of a group of diseases that are characterized by reduced bone marrow activity leading to a decrease in circulating cells of various hematopoietic lineages. The mechanisms involved in aplastic anemia are thought to include stem cell damage, abnormalities of the hematopoietic microenvironment, as well as inhibition of hematopoiesis by the immune system. There is growing support for the view that aplastic anemia can be mediated by impaired T cell function (12–16). Regulatory effects of mediators released by T lymphocytes, especially IFN-γ and tumor necrosis factor alpha (TNF-α), have been discussed in this context (17–19). In other reports, T cell–mediated inhibition of bone marrow activity was demonstrated to be MHC restricted, suggesting the involvement of specific cell/cell interactions in bone marrow suppression (20, 21).

Here we describe T cell clones that were established from peripheral blood of a patient with acquired severe aplastic anemia. A large proportion of these T cell clones showed stable coexpression of CD4 and CD8 molecules. We functionally characterized these T cell clones and investigated their ability to influence bone marrow differentiation in vitro.

Methods

Patient. The W.M. cell lines (described below) were derived from a 33-yr-old patient (HLA A11, A32; B35; Cw4; DR4, DRw12) who developed severe aplastic anemia 3 yr after a non-A, non-B hepatitis infection (white blood cell [WBC] count: 0.900/mm^3, Hb: 6.9 g/dl, platelets: 10.000/mm^3). Treatment with cyclosporin A, anti-thymocyte globulin, and methyl-prednisolon resulted in improvement of hematologic variables (WBC: 3.300/mm^3; Hb: 9.5 g/dl; platelets: 38.000/mm^3). T cell cloning was performed at diagnosis of severe aplastic anemia when the patient showed no symptoms of hepatitis and before he received any transfusion or other therapy. Patient W.M. gave an informed consent statement.

Cell cultures. PBL obtained after density centrifugation with Ficoll Hypaque (Pharmacia, Munich, FRG) were stimulated at 5×10^5 cells/ml with equal cell numbers of the lymphoblastoid B cell line Laz509 (irradiated, 60 Gray) in 24-well culture plates (Costar, Cambridge, MA). Culture medium was RPMI 1640 (Gibco Laboratories, Paisley, Scotland) supplemented with 2 mM L-glutamine (Gibco Laboratories), 100 U/ml penicillin (Gibco Laboratories), 0.1 mg/ml streptomycin (Gibco Laboratories), 10% heat-inactivated human serum, and pretested concentrations of IL-2–containing, T cell–conditioned medium. Cells were cultivated at 37°C, 7% CO_2, humidified atmosphere for 7 d and restimulated by adding 5×10^5 irradiated Laz509 stimulator cells to the same number of T cells. After two additional restimulations at day 14 and 21, respectively, T cells were cloned by limiting dilution

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1. *Abbreviations used in this paper:* BFU-E, burst-forming unit-erythrocyte; B-LCL, B-lymphoblastoid cell line; CFU-GM, colony-forming unit-granulocyte/macrophage; TCR, T cell receptor; TNF, tumor necrosis factor.

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with 0.3 T cells/well in 96-well V-bottomed culture plates (Costar) using Laz 509 and allogeneic PBL (each 5×10^4/well, irradiated) as feeder cells (see also Fig. 3, lane 4). Growing T cell clones were propagated in IL-2–containing culture medium and restimulated with feeder cells about every 10 d.

Transformation with EBV. Transformation of resting B cells was performed after preparation of PBL and removal of T lymphocytes by rosetting with sheep red blood cells (22). We used 10^7 cells with EBV-containing supernatant from the marmoset cell line B95/8.

Immunofluorescence analysis. Indirect immunofluorescence analysis of T cells was performed at 4°C as described (2). MAbs (23) specific for CD1 (19ThyIA8), CD2 (3PT2H9), CD3 (RW28C8), CD4 (12T4D11), CD5 (24T6G12), CD6 (3PT12B8), CD8 (21Thy2D3), IL-2 receptor (1HT4) and MHC class II (6) were from Dr. E. Reinherz, Dana Farber Cancer Center, Boston, MA, and were used in 1/1,000 dilution of mouse ascites. MAb Ti$^-\gamma$ A (24) was used in 1:3,000 dilution of ascites and MAb BMA-031 (a gift of Dr. R. Kurrle, Behring Werke, Marburg, FRG) was used at 10 μg/ml. After incubation of 10^6 cells in 0.1 ml MAb for 45 min, cells were incubated with 50 μl of fluorescein-labeled goat–anti–mouse Ig (Coulter Immunology, Hialeah, FL). For direct immunofluorescence T cells were incubated with phycoerythrine-labeled CD4 MAb (Leu 3a, Becton Dickinson & Co., Mountain View, CA) and fluorescein-labeled CD8 MAb (CD8A, Coulter Immunology). Cell-bound immunofluorescence was determined in an Epics C cell sorter (Coulter Immunology).

Cytotoxicity assay. Cytotoxicity was determined by standard methods using ^{51}Cr-labeled EBV-transformed B cell lines as target cells (2) (Laz156: HLA A2, 3; B7, 40; DR2, 4. Laz461: HLA A29, w23; B7. Laz509: HLA A2, 25; B 13, w38; Cw6; DR7. M7: HLA A3; B7, w6; DRw8, w25. Alex: HLA A2, 28; B27, w4; Cw2. Gerlach: HLA A1, 29; B37, w44; Cw6). 2×10^3 target cells/well were incubated at different E/T ratios with T cells for 4 h. In blocking experiments T cells were preincubated with T cell–specific MAbs, and target cells with MHC gene product–specific MAbs for 30 min. T cell–specific MAbs were the same as used for immunofluorescence analysis. MHC-specific MAbs were W6/32 (class I, Sera Laboratories, Sussex, England), 9/49 (class II, E. Reinherz), Leu10 (anti-DQ, Beckton Dickinson & Co.), D1-12 (a-DR), and B7-21 (a-DP) (both MAbs kindly provided by D. Charron, Paris, France). Specific lysis was determined as follows: Percent specific lysis = 100 × [(experimental release - spontanous release)/(maximum release - spontanous release)].

CFU granulocyte/macrophage (CFU-GM) assay. Bone marrow was obtained by aspiration from the iliac crest. Mononuclear cells were prepared by density centrifugation with Ficoll-Hypaque, washed, and enriched for progenitor cells by an immune rosetting technique as described (17, 25). Cultures were incubated at 37°C, 5% CO_2 in humidified air. At day 14 of culture, agar overlayers were removed from underlayers by agitation and dried onto glass slides. To examine the effects of T cells and their supernatants on myelopoietic colony growth, T cells were washed twice and were incorporated at 2.5×10^4 and 2.5×10^5 cells/ml into agar overlayers. Supernatants were conditioned by WM2 for 72 h and were added at 10 and 20% (vol/vol) to the overlayer cultures.

Burst-forming unit erythrocyte (BFU-E) assay. Erythrocyte bursts were grown in triplicate in 1-ml cultures of Iscoe's modified Dulbecco's medium (IMDM) containing 0.9% methylcellulose, 30% FCS, 0.9% deionized BSA, 2×10^{-4} M 2-mercaptoethanol, 2 U/ml erythropoietin (Step III, Connaught Laboratories, Swiftwater, Ontario). Cells (purified progenitor cells as described above) were plated at 5×10^3/ml. Late hemoglobizing bursts were scored as BFU-E at 14 d of culture.

IFN-γ and TNF-α determination. An IFN-γ–specific RIA was obtained from Cistron Technologies, Pine Brook, NJ. Sensitivity was shown to be 5 U/ml; no crossreactivity was apparent with type I IFNs. A TNF-α–specific RIA was kindly provided by Dr. G. Lambelin, IRE-Medgenix, Fleurus, Belgium. Sensitivity was shown to be 0.1 ng/ml TNF-α at 10% trace binding inhibition. IFN-γ was detected 48–72 h after specific T cell stimulation, TNF-α at 12–24 h after initiation of stimulated T cell cultures.

DNA isolation and Southern blotting. Genomic DNA was isolated from 10^7 cells by lysis in 0.5% SDS followed by phenol extraction as described (26). 15 μg DNA was digested overnight with 60 U of Eco RI, Hind III, Bam HI, and Sca I, respectively, (Boehringer Mannheim Biochemicals, Mannheim, FRG) and size-separated in 0.8% agarose gels. DNA was transferred to gene screen membranes (Gibco Laboratories) according to the manufacturer's instructions and hybridized with a [^{32}P]dCTP (New England Nuclear, Dreieich, FRG) -labeled 0.7-kb fragment of the pc_β REX plasmid (27) at 42°C using 50% formamid (28). Membranes were washed at 68°C and exposed to Kodak XAR films.

Results

Expansion of CD4$^+$/CD8$^+$ peripheral blood T lymphocytes. PBL from a patient (W.M.) with severe aplastic anemia were stimulated in vitro with the allogeneic irradiated B-lymphoblastoid cell line (B-LCL) Laz509. After in vitro expansion that

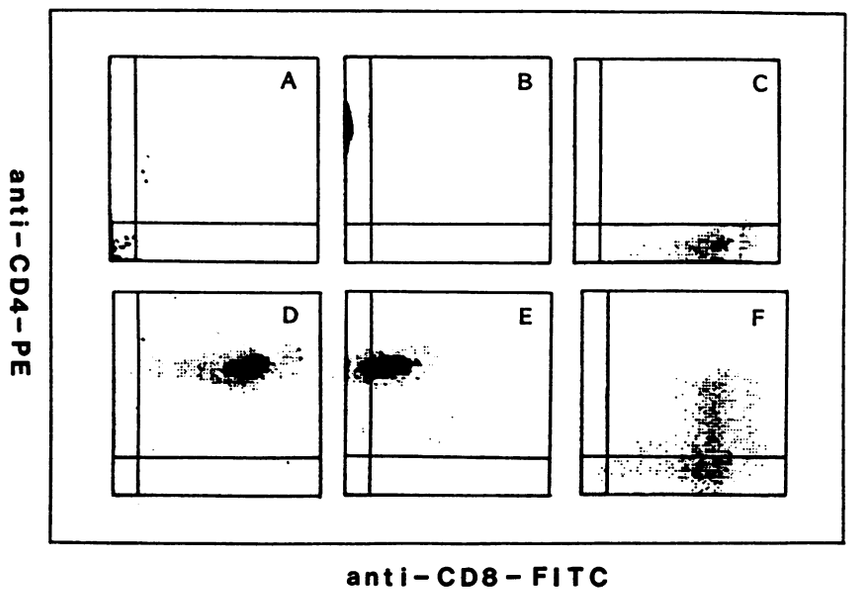

Figure 1. CD4/CD8 two-color immunofluorescence. WM2 cells were incubated with, respectively, FITC- and PE-labeled mouse-Ig (*A*), anti-CD4-PE (*B*), anti-CD8-FITC (*C*), and anti-CD4-PE plus anti-CD8-FITC (*D*), and fluorescence determined on an EPICS cell sorter. In panels *E* and *F* cells were incubated with unlabeled CD8 (*E*) and CD4 (*F*) MAbs, respectively, before labeling with CD4-PE plus CD8-FITC MAbs.

included three cycles of restimulation, responder cells were cloned by limiting dilution at 0.3 cells/well (60 wells). Subsequent phenotypic analysis showed that out of 18 independently derived T cell clones, 2 were $CD4^+$ and 8 T cell clones were $CD8^+$. Unexpectedly, eight additional T cell clones were > 90% reactive with both CD4 and CD8 MAb, as determined by indirect immunofluorescence. Coexpression of CD4 and CD8 molecules on these eight T cell clones was confirmed by two-color immunofluorescence analysis using directly labeled CD4 and CD8 MAb, which is shown in Fig. 1 for one representative T cell clone (WM2). Binding of directly labeled MAb was specifically inhibited by addition of unlabeled CD4 MAb (Fig. 1 *F*) and CD8 MAb (Fig. 1 *E*), respectively. Further phenotypic analysis (shown in Fig. 2) demonstrated that $CD4^+/CD8^+$ T cell clones expressed CD2, CD3, CD5, and CD6 molecules, receptors for IL-2 (CD25), and MHC class II antigens (Ia). CD1 molecules, which are invariably expressed on $CD4^+/CD8^+$ cortical thymocytes and some $CD4^+/CD8^+$ T lymphoma lines, were not detectable on these T cell clones (Fig. 2). Using a MAb specific for an invariant epitope of the TcR-α/β (BMA031) and a MAb specific for the TcR-γ/δ expressed on 2 of 3 TcR-γ/δ T lymphocytes (Ti-γA), respectively, it was found that $CD4^+/CD8^+$ T cell clones expressed an α/β heterodimer. This was confirmed by immunoprecipitation with the β chain–specific MAb β-F1 (29) (not shown).

In vitro growth of $CD4^+/CD8^+$ T cell clones was strictly dependent on IL-2 and restimulation with irradiated allogeneic feeder cells. In the absence of exogenous IL-2, T cell growth stopped within 2 d. Responsiveness to IL-2 lasted for ~ 15 d after restimulation, and could be reinduced by addition of irradiated feeder cells. These characteristics indicate that the $CD4^+/CD8^+$ T cell clones described here were not derived from an autonomously proliferating transformed T cell population. Coexpression of CD4 and CD8 molecules on W.M. clones was stable for > 9 mo of continuous expansion in culture, and did not change when T cell clones were grown in recombinant IL-2 or IL-4, respectively, instead of T cell–conditioned medium.

We reanalyzed the proportion of $CD4^+/CD8^+$ T lymphocytes during in vitro expansion in bulk culture of W.M. cells at different time points in the initial cell expansion. Thus, when T lymphocytes of the patient at day 7 of in vitro culture were analyzed by two-color immunofluorescence, 10% of $CD4^+/CD8^+$ T cells were detected. After further in vitro expansion, the amount of $CD4^+/CD8^+$ T cells increased up to 25% within the next 4 wk. In marked contrast to lymphocytes obtained and activated at the time of symptomatic disease, no increase in the number of $CD4^+/CD8^+$ cells was observed when T lymphocytes obtained during remission were stimulated and expanded in vitro (not shown).

Analysis of TCR gene rearrangements by Southern blot hybridization. TCR β chain gene rearrangements were analyzed to investigate whether CD4/CD8-coexpressing T cell clones established from the patient with aplastic anemia represented distinct clonal populations or whether these clones possibly descended from a single T cell. As shown in Fig. 3 *A*, when five $CD4^+/CD8^+$ T cell clones were analyzed by Southern blotting, they showed a germline configuration after cleavage of DNA with Hind III and hybridization with a TCR β chain constant region probe (lanes *1–4*, and *7*). Using Eco RI, we found in all five clones two identically rearranged bands of 13.0 and 8.0 kb, in addition to a 4.0-kb germline fragment (Fig. 4 *B*, lanes *1–4*, and *7*). When two clones (WM3, WM16) were further analyzed by using Bam HI and Sca I, an identical pattern of restriction fragments was observed (not shown). Two $CD4^-/CD8^+$ T cell clones from the same donor (Fig. 3, lanes *5* and *6*) with germline configuration after Hind III treatment showed different restriction fragments than $CD4^+/CD8^+$ clones from W.M. when Eco RI was used. A $CD4^-/CD8^+$ clone from a healthy donor (Fig. 3, lane *8*) investigated in parallel also presented with a different pattern of restriction fragments. These data suggest a clonal origin of the various $CD4^+/CD8^+$ T cell cultures.

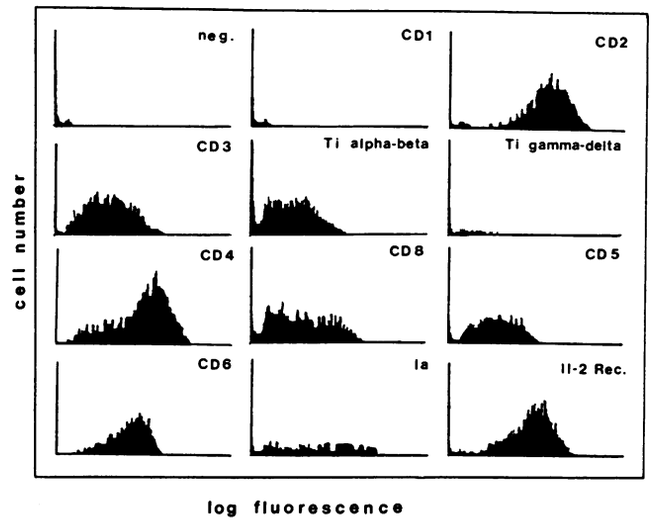

Figure 2. Phenotype of WM2. Indirect immunofluorescence of T cell clone WM2 using various MAbs as indicated was investigated on an EPICS cell sorter.

Cytotoxic activity of $CD4^+/CD8^+$ T cell clones. To characterize the cytotoxic activity of $CD4^+/CD8^+$ T cell clones we used a panel of ^{51}Cr-labeled B-LCLs as target cells in standard cytotoxicity assays. As shown for T cell clone WM2 in Fig. 4 *A*, the allogeneic B cell line M7 was strongly killed. In contrast, several other allogeneic cell lines, including Laz156 and Laz461, were not lysed. In addition, an intermediate level of cytotoxicity was observed towards a number of different B-LCLs, including Laz509. Other $CD4^+/CD8^+$ T cell clones from patient W.M. exhibited a similar pattern of cytotoxicity as WM2. Since these clones seemed to exhibit a broad cytotoxic specificity, we defined whether they might exert autocytotoxic activity. To this end, an autologous B-LCL was established by EBV transformation and was used as target in cytotoxicity assays. As shown in Fig. 4 *B*, autologous target cells were also killed by WM2 with an intermediate efficiency. In contrast, WM4, which is representative for two $CD4^-/CD8^+$ T cell clones derived from the same patient (shown in Fig. 4 *C*), did not exhibit cytotoxicity against autologous targets.

Blocking of cytotoxicity with MAb. To determine the MHC restriction of T cell clones coexpressing CD4 and CD8 molecules and in order to test whether this autocytotoxic activity with intermediate efficiency involves the TCR and MHC target structures, blocking experiments were performed. As shown in Fig. 5, preincubation of effector cells with MAbs specific for CD3, CD4, and MHC class II molecules, but not MAbs specific for CD8 or class I molecules, inhibited the cytotoxic activity of T cell clone WM2. In addition, cytotoxicity towards autologous targets was blocked by CD3 and MHC class II–specific MAb. This suggested that class II molecules were recognized as

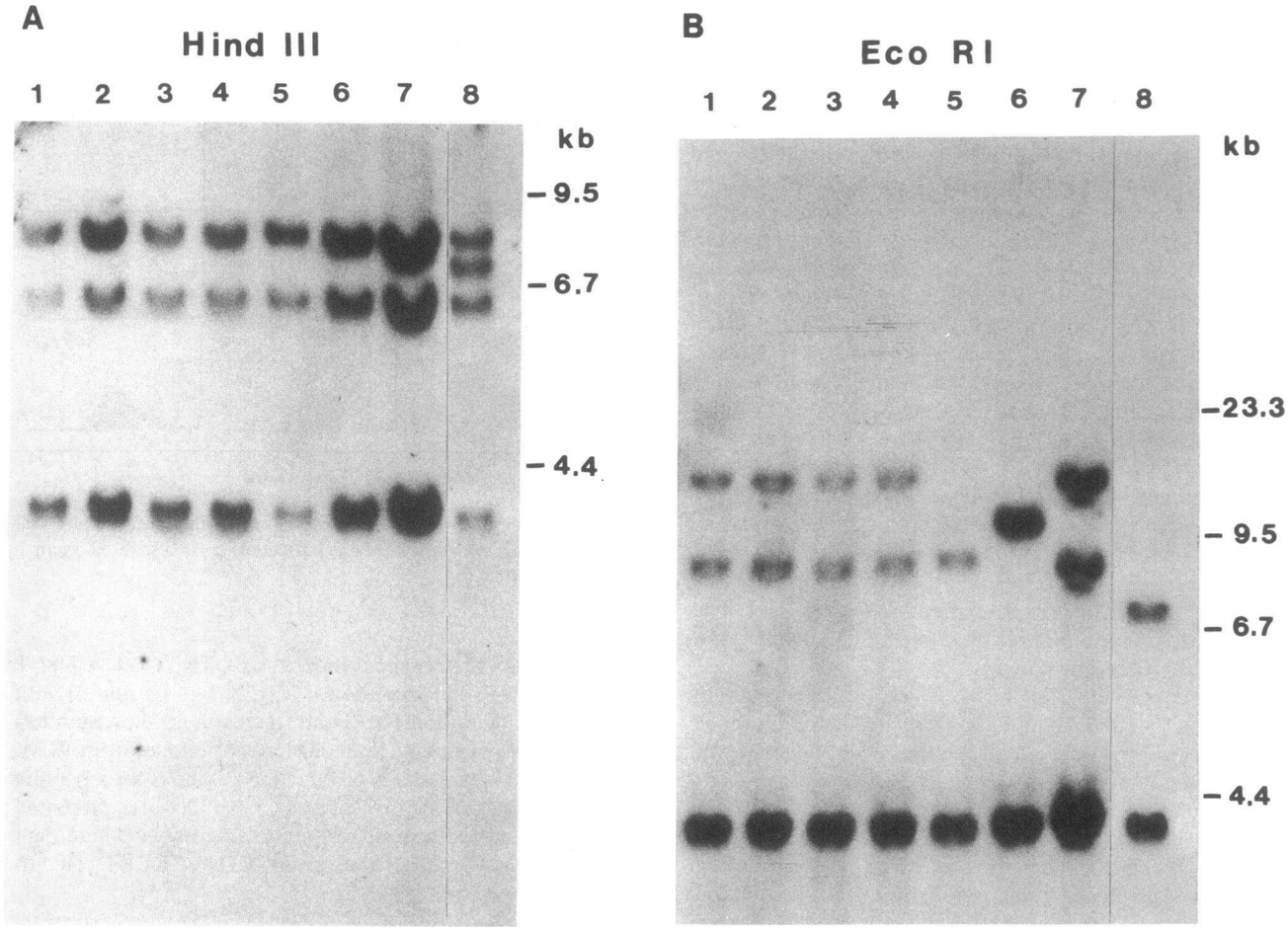

Figure 3. Southern blot analysis of TCR gene rearrangements. DNA from T cells was digested with Hind II (*A*) and Eco RI (*B*), respectively, transferred to a membrane, and hybridized with a TCR-cβ probe. DNA size was determined by comparison with a Hind III digest of λDNA. T cell clones were: (*A*) (lane *1*) WM17 ($CD4^+/CD8^+$), (lane *2*) WM16 ($CD4^+/CD8^+$), (lane *3*) WM5 ($CD4^+/CD8^+$), (lane *4*) WM3 ($CD4^+/CD8^+$), (lane *5*) WM8 ($CD4^-/CD8^+$), (lane *6*) WM4 ($CD4^-/CD8^+$), (lane *7*) WM2 ($CD4^+/CD8^+$), (lane *8*) C3F2 ($CD4^-/CD8^+$, from a different donor than W.M.).

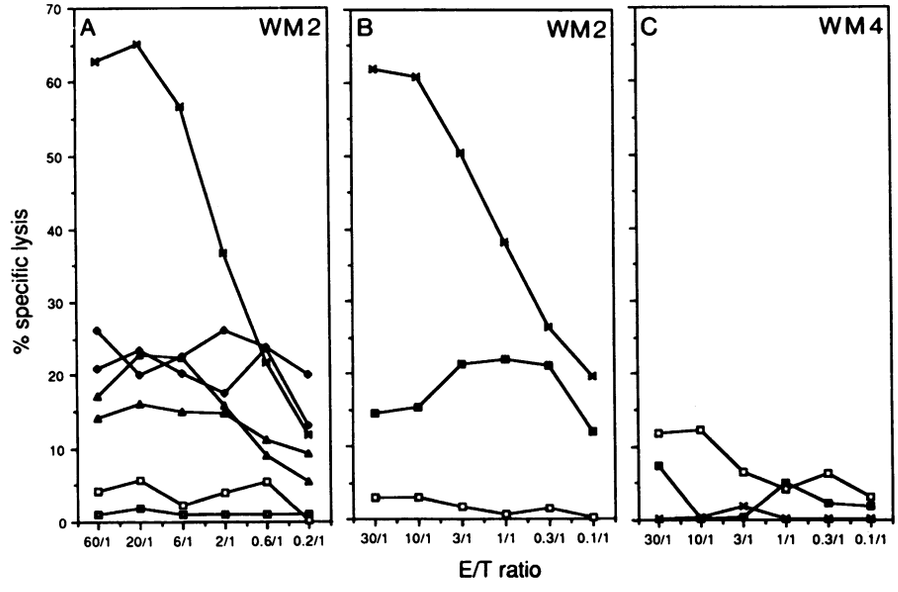

Figure 4. Analysis of cytotoxic activity. 10^3 ^{51}Cr-labeled target cells [Laz156 (▫), Laz388 (♦), Laz461 (□), Laz509 (◇), M7 (×), Alex (▲), Gerlach (△), autologous B-LCL (■)] were incubated with different numbers of effector cells in a 4-h cytotoxic assay. Effector cells were: (*A* and *B*, two individual experiments): WM2 ($CD4^+/CD8^+$) and (*C*, experiment in parallel to *B*): WM4 ($CD4^-/CD8^+$). Cytotoxicity is shown as percent specific lysis, which was determined from triplicates as described in Methods.

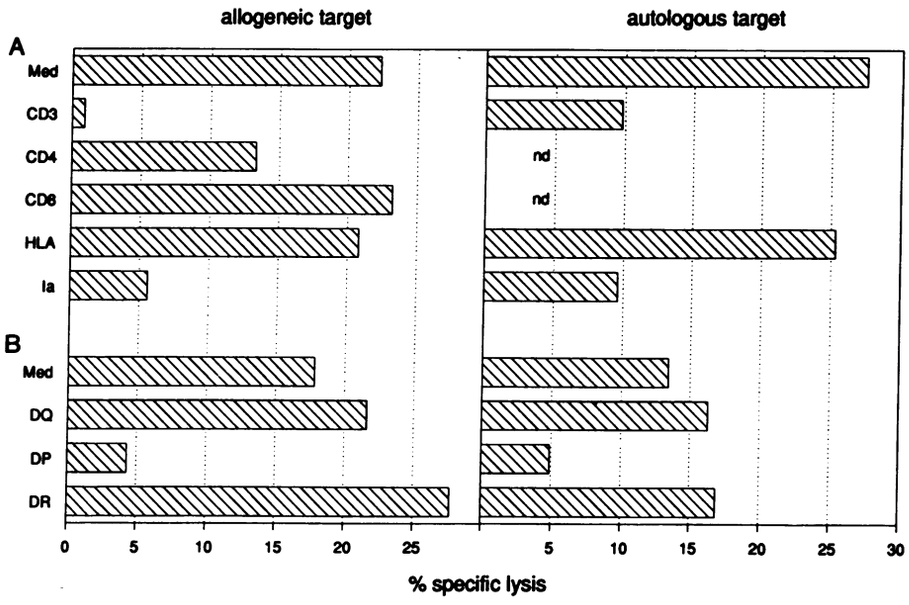

Figure 5. Blocking of cytotoxicity. 4×10^3 WM2 cells were incubated with 2×10^3 ^{51}Cr-labeled M7 and autologous B-LCL, respectively, in medium alone or with the addition of indicated MAb. Cytotoxicity as shown in percent specific lysis was determined from triplicate cultures after 4 h of incubation.

target structures in a TCR-dependent reaction. To further characterize the fine specificity of WM2, MHC class II subregion–specific MAbs were used in blocking experiments. As shown in Fig. 5, autocytotoxicity was inhibited by a MAb specific for DP-encoded class II molecules, whereas MHC-DR and -DQ–specific MAbs had no inhibitory effects. Similar experiments using the allogeneic M7 cell line as target demonstrated an identical MHC-DP specificity of WM2.

Effects of WM2 on autologous CFU-GM and BFU-E growth. The finding that autocytotoxic CD4$^+$/CD8$^+$ T lymphocytes with homogenous TCR β chain gene rearrangements existed in peripheral blood of patient W.M. prompted us to investigate whether these cells could influence the differentiation of autologous bone marrow cells in vitro. To investigate this point, T cells or T cell–derived culture supernatants were added to cultures of CSF-stimulated progenitor-enriched autologous bone marrow cells. As shown in Table I, colony formation of bone marrow cells was strongly inhibited by T cell clone WM2 at day 14 of culture. Plating of progenitor cells along with 2.5×10^4 and 2.5×10^5 cloned T lymphocytes resulted in 75 and 93% inhibition of colony formation, respectively. This inhibition was dependent on the physical presence of T cell clone WM2 in bone marrow culture. Thus, when cell-free supernatants of WM2 were added in concentrations of 10 and 20% (vol/vol) to progenitor cells, no inhibitory effects were observed. The influence of the CD4$^+$/CD8$^+$ T cell clone WM2 was specific for autologous bone marrow differentiation, since colony formation of progenitor cells from an allogeneic donor was not inhibited by WM2 (Table I).

Production of IFN-γ and TNF-α by T cell clone WM2. IFN-γ has been reported to represent a potent inhibitor of myelopoietic colony growth (30, 31). In this regard we have previously described a T cell line (SMAA), established from a different aplastic patient, that inhibited progenitor cell maturation by releasing high concentrations of IFN-γ (17). To investigate further the mechanism of bone marrow suppression mediated by T cell clone WM2, we determined the amount of IFN-γ and TNF-α released by WM2, compared with various T cell clones and cell lines, including the cell line SMAA. Table II shows that IFN-γ production of WM2 is comparable to that found in other T cell clones, PHA-activated T cells, and B lymphoblasts (< 800 U/ml). Similarly, TNF-α was released by WM2 cells in amounts that were comparable to those detected in other T cell cultures. These findings argue against a role of IFN-γ and TNF-α in the suppressive activities that WM2 exerts towards autologous bone marrow progenitor cells.

Discussion

T lymphocytes coexpressing CD4 and CD8 molecules account for 0.5–8% of circulating peripheral blood T cells in normal human beings (11). An increased number of peripheral

Table I. Inhibition of Autologous (A) and Allogeneic (B) CFU-GM and BFU-E Formation by WM2 Cells and WM2-Derived Culture Supernatant

		Day 14 CFU-GM* per 2.5×10^3 purified progenitor cells		Day 14 BFU-E‡ per 5×10^3 purified progenitor cells	
WM2 cells	WM2 supernatant	A	B	A	B
	% vol/vol				
		87±4	131±5	39±3	64±4
2.5×10^4/ml	—	21±2	129±5	17±3	62±5
2.5×10^5/ml	—	6±2	129±4	2±2	62±2
—	10	80±5	130±5	39±4	65±3
—	20	79±4	131±5	38±3	64±3

Autologous (*A*) or allogeneic (*B*) bone marrow–derived hematopoietic progenitor cells were plated at 2.5×10^3 cells/well (CFU-GM assay) or 5×10^3 cells/well (BFU-E assay) in overlayers of a double-layer agar or in single-layer methylcellulose culture systems in the presence of *colony-stimulating factors or ‡erythropoietin and were enumerated at day 14 of culture. In some experiments, overlayers or single layers received 2.5×10^4 to 2.5×10^5 WM2 cells/ml or supernatant conditioned by WM2 for 72 h (10–20% vol/vol).

Table II. Production of IGF-γ and TNF-α by Different Cell Lines

Cell line	IFN-γ production	TNF-α production
	U/ml	*ng/ml*
SMMA	15,000	ND
Laz156	110	ND
Laz509	24	21
WM2	37	12
WM4	10	11
WM7	78	17+
WM9	70	10
WM15	40	12
PHA-T cells	67	23

IFN-γ and TNF-α were determined in cell culture supenatants that were derived from different cell lines for 48–72 h and 12–24 h, respectively.

$CD4^+/CD8^+$ T cells were only observed neonatally in the developing immune system (32).

Unexpectedly, several $CD4^+/CD8^+/CD3^+$ T cell clones were obtained from one patient with severe aplastic anemia after alloactivation of his peripheral blood T lymphocytes and their further expansion in vitro. It is possible that such cells had a growth advantage during in vitro expansion, and were, therefore, obtained at high frequency despite their normal abundance in peripheral blood. This view is supported by the observation that repeated in vitro culture of uncloned alloactivated peripheral blood T cells from the patient resulted in an increase in the proportion of $CD4^+/CD8^+$ T cells (up to 25%). Moreover, identical TCR β chain gene rearrangements in several $CD4^+/CD8^+$ T cell clones suggest that these cultures descended from one single clone. The identical target cell specificities of the $CD4^+/CD8^+$ T cell clones support this view. Although clonality is very likely, TCR α chain gene rearrangements as well as V_β gene usage have not been investigated so far.

Transient coexpression of CD4 and CD8 can be induced by stimulating normal $CD4^+/CD8^-$ peripheral blood T lymphocytes with high doses of mitogen and T cell–derived supernatants (33). Moreover, $CD4^+/CD8^+$ T cell clones were obtained earlier using in vitro conditions where T cell growth was strictly dependent on IL-4 (addition of rIL-4 and rIL-2 MAb) (34). In the present experiments, however, the phenotype of WM2 was stable for several months of in vitro expansion and did not change whether the cells were grown in IL-2 or IL-4 or T cell–conditioned medium (not shown). Moreover, the fact that parallel establishment of T cell cultures from this patient resulted on one hand (symptomatic disease) in 25% of $CD4^+/CD8^+$ T lymphocytes, but on the other hand (remission) did not yield double positive T cells, argues against an influence of culture conditions such as exogenous lymphokines or mitogens on coexpression of CD8 with CD4.

Functional properties of $CD4^+/CD8^+$ T cells are less well defined. As reported by others, $CD4^+/CD8^+$ T cell clones produce similar patterns of lymphokines, although in different quantities, when compared with T cell clones of other phenotypes (35). They were also shown to exhibit helper cell function and MHC-unrestricted cytotoxicity. Further analysis of a $CD4^+/CD8^+$ T cell clone suggested that both CD4 and CD8 molecules were involved in signal transduction in the course of T cell activation (36, 37). The $CD4^+/CD8^+$ T cell clones characterized here were found to exhibit cytotoxic activity towards a number of different B-LCLs. Characteristicly, one B-LCL that was not used for allostimulation was lysed very efficiently, and some other target cell lines were lysed with less efficacy, especially at high E/T ratios. Importantly, one representative clone, namely WM2, exhibited a similar kind of intermediate cytotoxicity towards autologous B lymphoblasts. The difference in lytic activity towards different target cells may be due to differential expression of molecules that, besides the TCR, are involved in E/T cell interaction (i.e., CD2, CD11a, CD54, CD58; references 38, 39). Whether, in addition, binding of T effector cells to other T effector cells expressing autoantigens may lead to inhibition of autocytotoxicity towards labeled target cells (cold target cell inhibition) is at present not known.

Interestingly, hypogammaglobulinemia with regard to IgG (6.4 g/l [normal 8–18 g/liter]) and IgM levels (0.4 g/liter [normal 0.6–2.5 g/liter]), but not for IgA (3.0 g/liter [normal 0.9–4.5 g/liter]), existed in patient W.M. However, whether this in vivo symptom bears any relationship to the fact that $CD4^+/CD8^+$ T cells exhibited lysis toward autologous B lymphoblasts in vitro remains unclear.

Autocytotoxicity as observed here is dependent on specific antigen recognition by the TCR since it was blocked by $CD3^-$ and MHC class II–specific MAbs. Using MAbs in blocking studies, cytotoxicity of WM2 was found to be restricted to MHC-DP molecules. Involvement of MHC class II gene products in autologous target cell recognition is in agreement with previous reports (40, 41), although autoreactive T cell clones specific for MHC-DP molecules have not been reported so far. Note that $CD4^-/CD8^+$ T cell clones from the same donor showed no cytolytic activity towards autologous targets (Table III).

Earlier reports demonstrated that bone marrow activity is modulated by subpopulations of T lymphocytes exhibiting either enhancing or inhibitory effects on progenitor cell growth in vitro (12–18, 42–44). In this regard, IFN-γ (30, 31) and

Table III. Inhibition of Cytotoxicity of T Cell Clone WM2 with MAb

		Target cell	
	MAb	Autologous B-LCL	Allogeneic B-LCL (M7)
A	None	27,5	22,3*
	CD3	9,8	0,9
	CD4	ND	13,3
	CD8	ND	23,2
	HLA	25,3	20,8
	Ia	9,6	5,6
B	None	13,4	17,8
	DQ	16,3	21,6
	DP	4,9	4,3
	DR	16,9	27,6

2×10^3 ^{51}Cr-labeled autologous B lymphoblasts were incubated with 6 $\times 10^3$ WM2 cells in the presence of different MAb. (*A*) CD3, ascites dilution 1/300; a-HLA (class I), a-Ia (class II), ascites 1/100. (*B*) MHC-DR, ascites 1/100; a-DQ, ascites 1/450; a-DP, supernatant 1/10. Cytotoxicity was determined from triplicates after 4 h of culture.
* Percent specific lysis.

TNF-α (19) were identified as inhibitory mediators, and the suppressive activity of activated T lymphocytes could be attributed, at least in some cases, to the release of IFN-γ by T cells. The possible role of IFN-γ in the pathogenesis of aplastic anemia has been challenged (45) in another report. This issue, therefore, remains controversial but could also be taken as an indicator for the heterogeneity of this disease with regard to underlying pathomechanisms. Genetic restriction was previously observed, suggesting specific cell/cell interaction underlying bone marrow inhibition (46). Interestingly, T cell clone WM2 displayed inhibitory activity towards autologous but not unrelated allogeneic bone marrow cultures. The inhibitory effect of WM2 was dependent on physical cell/cell interaction and could not be attributed to enhanced production of mediators such as IFN-γ and TNF-α. This finding is not surprising since double-positive T cell clones have been reported to secrete rather low amounts of IFN-γ (and IL-2) when compared with "conventional" T lymphocytes (35).

A linkage between bone marrow failure in aplastic anemia and impaired T cell function has been suggested from several studies (12, 13). Increased numbers of $CD4^-/CD8^+$ circulating as well as marrow T cells exhibiting suppressive activity on progenitor cells were reported to occur in patients with aplastic anemia (14–16). In some cases, impaired bone marrow differentiation was linked to an enhanced production of IFN-γ (17, 18, 47). On the other hand, increased proportions of genetically restricted $CD4^-/CD8^+$ T lymphocytes (20) and autocytotoxic T cells (21), respectively, were described.

Taken together, a clonal population of autocytotoxic $CD4^+/CD8^+$ T cells that suppresses autologous bone marrow cell differentiation in vitro was expanded from peripheral blood of one patient with aplastic anemia. The frequency of circulating $CD4^+/CD8^+$ T cells was, however, not enhanced in this patient, which may challenge the relevance of those double-positive cells for the clinical symptoms in patient W.M. Nevertheless, a clonal population, even if it accounts for $< 1\%$ in peripheral blood, would still have to be considered a highly frequent effector cell population expressing a single specificity.

Whether the $CD4^+/CD8^+$ T lymphocytes identified here are of relevance with regard to bone marrow failure in patient W.M. remains speculative. A possible in vivo significance of such a cell population would require investigations of a larger group of patients before firm conclusions could be drawn. However, given the heterogeneity of this disease, such a possibility can not be excluded. The fact that $CD4^+/CD8^+$ T cells were not expanded during remission and that the clinical symptoms of patient W.M. improved upon immunosuppressive therapy would support such a view. Moreover, with regard to a possible pathomechanism, expression of MHC-DP molecules was reported to occur on hematopoietic progenitor cells (48, 49); therefore, these cells could serve as targets for DP-restricted autocytotoxic T cells.

CD4/CD8 coexpression is found during thymic ontogeny, particularly on immature cortical thymocytes. $CD4^+/CD8^+$ thymocytes are believed to represent an intermediate stage of T cell development, and elimination of thymocytes expressing high affinity receptors for self antigens during ontogeny likely represents one mechanism underlying T cell tolerance (4–7). Therefore, one hypothesis to explain the circulation of autoreactive $CD4^+/CD8^+$ T lymphocytes in patient W.M. would be that these cells had escaped clonal deletion in vivo. The fact that these double-positive T cells did not express CD1 molecules would, however, not be in line with accepted models of thymic maturation (1). An alternative interpretation could be that $CD4^+/CD8^+$ T cells represent a physiologically separate lineage of mature circulating T cells and that the execution of their autoaggressive potential is due to reduced activity or even loss of other regulatory cell populations.

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